Practical Applications of Rolling Circle Amplification of DNA Templates

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Introduction

Rolling circle amplification was initially described as the mechanism by which a variety of viruses replicate their circular genomes[1-5]. Since that time, a number of *in vitro* applications of the rolling circle mechanism have been described[6]. The three basic *in vitro* forms of rolling circle amplification (RCA) can be distinguished by the number of primers used in a reaction (see Figure 1). In the linear form of RCA, a DNA circle is amplified by polymerase extension of a single complementary primer in an isothermal reaction. Up to 10^5 tandemly-repeated, concatemerized copies of the DNA circle are generated by each of these primers. The so-called exponential form of RCA uses a second DNA primer of identical sequence to the DNA circle. The third RCA format, multiply-primed RCA, employs a mixture of random primers and the same highly processive polymerase used in linear RCA. Each of these RCA formats is associated with unique sets of applications, which will be described in more detail below.

Linear RCA generates a single, amplified product linked to an initiating primer. Incorporation of modified bases into this product, or hybridization of this product with labeled oligonucleotides, provides a powerful method of signal amplification. Several recent studies demonstrated the utility of linear RCA signal amplification on DNA and protein microarrays. On DNA arrays, RCA gives up to a 10,000-fold increase in signal over hybridization of a fluorescently-labeled probe; in fact, as few as 150 DNA molecules bound to the surface of microarrays can be detected using RCA[7]. Since the RCA reaction exhibits linear kinetics, the amount of DNA target molecules can

be quantified with a dynamic range of 4 orders of magnitude[7]. These properties of RCA were combined with PCR ligation on universal oligonucleotide microarrays by Ladner *et al.*[8] to permit the detection and quantification of single mutations within a pool of 100 wild-type alleles.

Schweitzer *et al.*[9] showed that linear RCA could be employed for signal amplification of proteins in either microarray or microtiter plate format. This was accomplished by attachment of the 5' end of an RCA primer to an antibody, which resulted in the formation of RCA products attached to the antibody. This form of RCA, termed "immunoRCA", enabled a 100-fold increase in antigen-detection sensitivity in a microtiter ELISA assay[9]. Protein analytes captured on microarrays have also been measured in a similar fashion; in this format, RCA products are labeled with fluorescent probes and detected on a microarray scanner. This adaptation of RCA was also used to measure 75 different cytokines simultaneously on microarrays with high specificity and a sensitivity that was 1000-fold better than direct detection with a fluorescently-labeled antibody[10]. Similarly, linear RCA was applied to the measurement of IgE in serum samples to multiple allergens immobilized on a microarray and demonstrated a clinical sensitivity and specificity that was equal or superior to conventional immunoassays[11]. ImmunoRCA has also been successfully applied to the immunohistochemical detection of a variety of cell surface antigens as well as intracellular molecules within routinely fixed specimens, resulting in an increased sensitivity of antigen detection over conventional staining methods[12].

A two-primer RCA system achieves isothermal, exponential amplification. Exponential RCA has been applied to mutation detection through use of a linear DNA probe that binds at both of its ends to contiguous regions of a target DNA, followed by circularization by DNA ligase. Thomas *et al.*[13] demonstrated sensitivity of 10 target molecules and 10^7-fold amplification in 1 hour in a homogenous closed-tube format using open circle probes, exponential RCA, and Amplifluor detection probes. Faruqi *et al.*[14] described a solution-based, microtiter plate method for SNP genotyping directly from genomic DNA based upon allele discrimination by ligation of open circle probes followed by RCA of the signal using fluorescent primers. Genotyping without the need to preamplify the target was demonstrated using only 1 ng of genomic DNA.

Target amplification by linear RCA is limited to circular nucleic acids, such as circular viruses, plasmids, and circular chromosomes. In multiply-primed RCA (MP-RCA), the use of short random primers annealed to a circular or linear double or single-stranded DNA template generates multiple replication forks. RCA proceeds by displacing the nontemplate strand, resulting in the formation of products consisting of tandem copies of the circle. Since the priming is random, synthesis proceeds on both strands, resulting in a double-stranded product. Using random primers and the highly processive ϕ29 polymerase, Dean e*t. al.*[15] demonstrated that circular DNA templates can be amplified 10,000-fold in a few hours. The amplified product can be used directly for DNA sequencing, thereby removing the need for lengthy growth periods and traditional DNA isolation methods. Since the product is double-stranded, DNA sequencing of either strand is possible; furthermore, the product can be digested with restriction endonucleases for use in a number of different cloning methods.

A commercial kit, TempliPhi™, has recently been introduced by Amersham Biosciences, which employs MP-RCA for the generation of sequencing templates from small amounts of plasmid, from either purified or unpurified forms. The DOE Joint Genome Institute optimized conditions for use of MP-RCA in sequence template generation, and has enjoyed significantly increased pass rates and read lengths as a result. The institute is now routinely producing over 1 billion high quality bases of sequence per month based on these protocols (PR, personal communication).

In addition to its use for sequencing templates, MP-RCA has a variety of other applications in the field of molecular biology and genetic engineering. This chapter will explore a variety of these applications in addition to sequencing applications.

Sequencing Applications

Sequencing from rolling circle amplification templates can be accomplished from a variety of inputs (such as *E. coli* plasmids, cosmids, fosmids, BACs, viruses and *S. cerevisiae* plasmids). The protocols share several basic steps, irrespective of input type:

1. Sample denaturation: Sample denaturation is accomplished by heating the sample to ~95 °C for a short period of time. The DNA sample, either single- or double-stranded, can be purified or in whole cells / virus. When amplifying from bacterial cells the heating time should not exceed 5 minutes, in order to avoid release of bacterial chromosomal DNA. When denaturing from whole cells or virus it is advisable to centrifuge the samples to remove debris.

2. Rolling circle reaction: A small amount of denatured sample is added to a reaction mix containing ϕ29 DNA polymerase, random primers and nucleotides, and the reaction is allowed to proceed at 30 °C for several hours. While 4-6 hours is suggested in the TempliPhi™ product literature, we have found that an overnight incubation (16 hours) yields more consistent and reliable results in a high-throughput setting.

3. Enzyme inactivation: The DNA polymerase is inactivated by heating to 65 °C for 10 minutes. This step inactivates the exonuclease activity of the enzyme and prevents it from potentially interfering with the sequencing reaction, since the product will be used directly for DNA sequencing without prior purification.

4. Sequencing: Since the MP-RCA reaction runs essentially to completion, the nucleotides in the reaction mix are sufficiently exhausted so as not to interfere with the subsequent sequencing step. Therefore, the MP-RCA product can be used directly for sequencing (If desired, excess terminators can be removed by standard ethanol precipitation or by using magnetic bead protocols[16]). We have used amplified samples in sequencing reactions employing either ET (Amersham Biosciences) or Big Dye (Applera) terminator chemistry.

Due to inherent differences in the nature of inputs, the success of the process can vary significantly according to input type. Below we describe our experiences with amplification and sequencing from a variety of input types.

Plasmid template input:

MP-RCA is ideally suited to producing sequence-ready plasmid templates from colonies, growing cultures or glycerol stocks. The procedure is essentially the same for each; a small amount of colony is picked into or a small aliquot of culture (1 μl) is added to denaturation buffer (10 μl)(100mMTris, .1mM EDTA) and the bacterial cells are gently lysed at 95 °C for 5 minutes and cooled to 4 °C. At the DOE Joint Genome Institute Production Sequencing Facility, template is routinely generated from glycerol stocks. The amplification procedure is readily amenable to high-throughput processes using robotic liquid handling devices and can be carried out in 384-well plates. The amplified products can be used directly in sequencing reactions with sufficient template per amplification for at least 20 reactions. An added benefit of this procedure is the production of nearly uniform concentrations of DNA in each well (Figure 2), thus increasing reproducibility and success rate of the downstream sequencing applications compared to other methods. Uniformity of template concentration is particularly important when sequencing on capillary instruments. Using this amplification process, the US Department of Energy Joint Genome Institute has produced over 500,000 lanes of sequence averaging approximately 90% pass rate and a 600bp average read length. This represents a significant improvement over the output obtained previously using purified plasmids (Figure 3).

S. cerevisiae input:

Yeast plasmids can be amplified and sequenced using essentially the same protocols used for *E.coli*. As with plasmids amplified from *E. coli*., the MP-RCA DNA product yield is highly uniform, which is important for capillary based sequencing. However, sequencing readlengths are generally lower than with *E. coli*. Modifications to the standard lysis procedure may improve results.

Phage input:

Lambda phage particles can be grown, lysed and amplified for sequencing. In our hands, a culturing step of picking phage plaques into LB/Maltose media containing XL-1 starter cultures and growing overnight in 1ml media in 96-well blocks produces the most consistent results. Phage cultures are then lysed at 70 °C for 15 minutes and spun at 4000 rpm for 20 minutes (in 96-well plates, Eppendorf model 5810 centrifuge) to pellet cell and coat debris. Aliquots of supernatant can then be amplified as for plasmids.

Large insert inputs:

Clones containing larger inserts such as cosmid, fosmid and BAC vectors have proven to be more difficult to amplify for use as sequencing templates. This is primarily related to lysis conditions that also release variable amounts of genomic *E.coli* DNA. Since amplification can also act on long linear templates, significant amplification of the *E.coli* genomic DNA can occur. This results in variable success rates when larger low copy vectors are used for this procedure. However, we have obtained usable sequence information, often with very good read lengths using the standard procedure. In addition, we have been able to increase success rates by performing modifications of standard alkaline lysis preps followed by standard MP-RCA on the purified product.

Molecular Biology Applications

Amplification of plasmids for restriction digestion:

The DNA produced by MP-RCA can be used for additional purposes such as restriction digestion. Because negative control MP-RCA amplifications can themselves produce DNA product (presumably through amplification of small amounts of contaminating DNA), restriction digestion may be necessary to confirm that the desired target has been amplified. As well, restriction digestion can be used to screen for the presence of the desired plasmid in transformants. Some optimization of reaction conditions may be necessary, as we have noted that restriction digestion of amplified DNA may give variable results depending on the enzyme used and temperature of incubation. An example is shown in Figure 4.

Transformation:

MP-RCA amplified DNA can be used in the transformation of both bacterial and eukaryotic cells. In our hands, transformation of *E. coli* using amplified DNA is approximately 1 order of magnitude more efficient than with unamplified negative control (recall that unamplified control contains plasmid from they lysed *E. coli*). The mechanism of this enhancement is unclear, but does not appear to be a general effect of the presence of amplified DNA since simple addition of amplified DNA to purified plasmid DNA (with a different selectable marker) does not increase the transformation efficiency of the plasmid (Predki, unpublished results).

We have found that plasmid DNA amplified by the MP-RCA procedure can also be used for efficient electroporation into eukaryotic cells. We employ a screen for cis-acting regulatory DNA by transforming a reporter plasmid into fertilized ciona intestinalis embryos. *Ciona intestinalis*, or sea squirts are a Urochordate that are sedentary marine filter feeders as adults, but exhibit many vertebrate characteristics in their embryonic stage. The embryos develop from fertilized eggs in approximately 24 hours to become free swimming tadpoles 1-2mm in size. After swimming for 1-3 days, they attach to a solid surface and metamorphosize into their adult form. *Ciona* is a model developmental organism and a complete cell fate map exists for the 2500 cell stage tadpoles.

We employ the embryos as a system to assay DNA for tissue specific gene regulatory activity. The reporter plasmid contains a minimal *Ciona* forkhead promoter linked to a lacZ reporter gene[17]. There is a multiple-cloning site about 200 bases upstream into which random or selected stretches of *Ciona* genomic DNA (300bp-4kb) can be inserted. Plasmids are then electroporated into fertilized eggs at the one cell stage, and embryos allowed to develop for 24 hours before being fixed and stained for lacZ activity. Embryos are allowed to develop for 24 hours when they are fixed and stained for lacZ activity. As shown in Figure 5, we obtain the same results when the DNA used for electroporation is produced by standard techniques (Qiagen midi-prep) or when plasmid DNA is produced from glycerol stocks by MP-RCA using Templiphi™.

Whole Genome Amplification:

Amplified genomic DNA can be used for several applications such as shotgun library generation, SNP genotyping, Southern blotting, RFLP, labeling of genomic DNA and DNA sequencing. (also see Richardson, Detter, & Hawkins[18]).

In one specific application, genomic amplification may provide an important route to generating sufficient quantities of DNA for genomic sequencing of microbes which are difficult to culture. We have demonstrated amplification of bacterial genomic DNA using the MP-RCA with ϕ29 polymerase (see Figure 6). Amplification can be applied to small amounts of isolated DNA or directly to cells. Sequence analysis of shotgun libraries produced from amplified bacterial genomic DNA indicates that the amplification is essentially random (unpublished results). We have also labeled amplified genomic DNA for use in microarray experiments. Figure 7 show a microarray image where amplified genomic DNA from a strain of *Xylella fastidiosa* was used as the target in a microarray experiment with probes designed from predicted open reading frames within the genome. Independent experiments have confirmed that identical results are obtained when genomic DNA is directly labelled (unpublished).

Dean *et. al.*[19], have recently demonstrated whole genome amplification of eukaryotic genomic DNA using what they term multiple displacement amplification. This method also makes use of the ϕ29 polymerase and random hexamers for priming, but amplification proceeds from long linear templates as opposed to circular ones. Amplification was achieved from different biological samples, including crude whole blood and tissue culture cells. In contrast to PCR-based methods of whole-genome amplification, this approach appeared to exhibit little amplification bias.

Summary

Since its recent implementation at one of the world's largest high-throughput sequencing centers, the utility of MP-RCA for DNA sequencing has been thoroughly validated. However, applications of this technology extend far beyond DNA sequencing. While many of these applications have been explored in this chapter, the future will undoubtedly add to this growing list.

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FIGURE LEGEND

Figure 1. **The 3 basic types of rolling circle amplification.**

(a) Linear rolling circle amplification. A DNA primer (black rectangle) hybridizes to a circular DNA and initiates the synthesis of multiple concatamerized copies by DNA polymerase. Direction of polymerization is indicated by the arrow.

(b) Exponential rolling circle amplification. A DNA primer (black rectangle) hybridizes to a circular DNA and and initiates the synthesis of multiple concatamerized copies by DNA polymerase. Subsequently, reverse primers (white rectangles) prime additional synthesis at each tandem repeat. Higher order priming (not shown) will also occur. Direction of polymerization is indicated by the arrow.

(c) Multiply-primed rolling circle amplification. A random DNA primer (gray rectangle) hybridizes to a circular DNA and initiates the synthesis of multiple concatamerized copies by DNA polymerase. Secondary non-specific priming events can subsequently occur on the displaced product strands of the initial rolling circle amplification step. Higher order priming (not shown) will also occur. Direction of polymerization is indicated by the arrow.

Figure 2. **Agarose image of rolling circle amplified sequencing template**. 200 ng DNA standard (left) and Lamda HinDIII size markers (middle) are apparent. Product was amplified from glycerol stocks of a pUC18-based genomic shotgun library. These products were heated to 95 ^{o} C, causing the high-molecular weight, highly branched amplified DNA to remain near the loading wells on this standard 1% agarose gel. Note the relatively uniform intensity of the DNA product.

Figure 3. **Impact of rolling circle templates on sequencing results.** Improvement in average sequencing quality (q20 bases per read) during 2001 (% improvement), during which the DOE Joint Genome Institute transitioned from plasmid templates to rolling circle templates. The sequence quality improvements parallel the implementation of and transition to rolling circle templates (%RCA). Overall, rolling circle templates have increased the production of high quality bases by an average of 50%.

Figure 4. **Restriction digestion of rolling circle amplified product.**

(a) Genomic shotgun 8-10 kb insert libraries were cloned into the vector pCUGIblu21 (see 4b).In order to assess the quality of the libraries, random colonies were amplified by rolling circle and 5 μl of the product was digested with the rare-cutter SWAI (New England Biolabs) in a 10 μl total volume under conditions recommended by the manufacturer. In the agarose image, the 2.3 kb vector band appears in all sample lanes (see restriction map in Figure 4b), and the size of the inserts can be estimated from the Lambda HinDIII standards (left and middle).

(b) Restriction map of the plasmid pCUGblu21 used to construct the 8-10kb library (Rod A. Wing and Tae-Jin Yang, Arizona Genomics Institute, University of Arizona, personal communication, rwing@ag.arizona.edu)

Figure 5. **LacZ activity of *Ciona intestinalis* 24 hour embryos viewed under a dissecting microscope.**

(a) Embryos transformed with the reporter vector alone (DNA purified via Qiagen protocols) show typical background staining in the mesenchyme cells (Richardson, unpublished results).

(b) Embryos transformed with a reporter vector with a 3kb region of *Ciona* genomic DNA inserted upstream of the promoter. DNA purified via Qiagen protocol.

(c) Embryos transformed with a reporter vector with a 3kb region of *Ciona* genomic DNA inserted upstream of the promoter. DNA produced by MP-RCA protocol.

Figure 6. **Agarose image of bacterial genome amplification.** Overnight cultures *of Xylella fastidiosa* (lanes 1-4) or *E.coli* (lanes 7-10) were subjected to MP-RCA according to standard protocols. Cultures were diluted by 1:1000 (lanes 4 & 10), 1:100 (lanes 3 & 9), 1:10 (lanes 2 & 8) or undiluted (lanes 1 & 7) prior to MP-RCA. 10% of the amplified DNA was run on a 1% agarose gel; these highly branched concatamers typically co-migrate with the 23kb lambda HIII band at the limit of resolution of a standard 1% agarose gel. Controls include MP-RCA amplified water (lane 5) and 125 ng and 250 ngDNA quantity standards (lanes 13 & 14 respectively)

Figure 7. **Use of fluorescently labeled amplified genomic DNA in a microarray experiment.** *Xylella fastidiosa* citrus strain genomic DNA was amplified by MP-RCA according to standard protocols, sheared to 2 kb fragments by a HYDROShear device (GeneMachines) and Cy-3dCTP labeled with Klenow polymerase. Labelled targets (from the amplified genomic DNA) were ethanol precipitated and used for glass slide hybridization. The image was made using labeled citrus genomic DNA used as the target in microarray experiments with 70 base oligomers as probes. The oligomers were designed based on predicted open reading frames from genomic sequence data. Results were essentially the same when using unamplified or MP-RCA amplified genomic DNA for labeling.